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Age-Dependent Response of the Rat Testes to Di(2-ethylhexyl) Phthalate

by Per Sjöberg*†, Nils Gunnar Lindqvist†, and Leif Plöen*

Spermatogenesis starts soon after birth in the Sprague-Dawley rat but is not fully established until about 56 days of age. When high oral doses of the plasticizer di(2-ethylhexyl) phthalate (DEHP) were given to rats of different ages, testicular damage was observed in immature but not in mature animals. Plasma concentration and urinary excretion data suggested that the gastrointestinal absorption of the DEHP-derived metabolite mono(2-ethylhexyl) phthalate was higher in young animals. Young rats were not more susceptible than older for repeated intravenous infusions of DEHP. It is suggested that the age-related difference observed in testicular response after oral administration of DEHP may be due to pharmacokinetic rather than tissue sensitivity differences. It is concluded that in assessing risks of testicular injuries in children exposed to DEHP, additional studies are required using species in which testicular development is more similar to that of humans.

Introduction

Di(2-ethylhexyl) phthalate (DEHP) is a commonly used plasticizer which has become widely distributed in the environment (*1–3*). Furthermore, blood and blood products may leach DEHP out of plasticized medical devices leading to inadvertent patient exposure during, e.g., hemodialysis and blood transfusions (*4,5*). As yet, exposure in humans is not known to be associated with any adverse effects. Administration of high oral doses of DEHP to rats have resulted in liver abnormalities and testicular damage (*6,7*). The effect on testes has been observed to vary with the age of the animal, younger rats being more sensitive than older ones (*8*). In this communication, possible mechanisms involved in this age dependency will be discussed.

Aspects of the Development of the Testis

Before going into age-related effects of DEHP it is necessary briefly to discuss some of the factors involved in the normal development of the testis and to define testicular development.

Testicular development and function are dependent on many factors (*9*). It is known that gonadotrophic hormones from the pituitary act on the Sertoli cells, either directly (FSH = follicle stimulating hormone) or via the Leydig cells (LH = luteinizing hormone); it has also been shown that the Sertoli cells are involved in the regulation of germ cell differentiation (*10,11*). The complicated cell–cell interactions within the testis are not fully understood (*12*).

The development of the testis in the Sprague-Dawley rat is morphologically well described. In the newborn rat the seminiferous cords contain only undifferentiated Sertoli cells and gonocytes. At about 10 days of age most gonocytes have differentiated into spermatogonia (*13*). The first primary spermatocytes appear at day 12 or 13, and formation of the occluding inter-Sertoli cell junctions takes place between 15 and 19 days of age (*14,15*). At 24 days the first meiotic divisions are observed (Fig. 1), and spermatids in the Golgi phase have been demonstrated at day 25 (*16*). At 44 days the first mature spermatids ("testicular spermatozoa") are observed, but not until about 56 days of age is the frequency of such late spermatids the same as in the adult testis (*17*). Up to 48 days of age a considerable number of exfoliated, more or less degenerated, testicular cells are found in the epididymis.

From day 20 to day 60 there is a 17-fold increase in testicular weight but only a 7-fold increase in body weight. Tubular diameter increases about 2.5 times during this period and total tubular length increases about 3 times (*17*).

Both testicular and plasma testosterone concentrations are low until about 50 days of age (*16,18*), except for a short postnatal period when testosterone levels are high, due to the influence of maternal gonadotrophins (*19*). The rise in testosterone is preceded by an increase in serum LH, which starts at about 30 days of age and reaches a maximum at about 50 days (*20*). The number of LH receptors shows a similar increase (*19*). FSH levels rise somewhat earlier (about day 20) and from 30 days of age FSH levels are fairly constant (*20*).

*Department of Anatomy and Histology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden.
†Department of Drugs, National Board of Health and Welfare, Uppsala, Sweden.

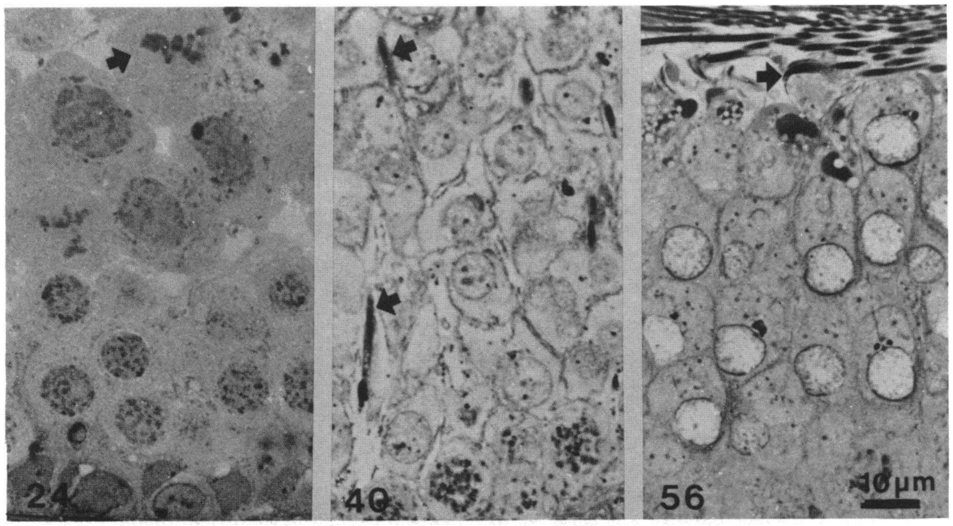

FIGURE 1. Light micrographs from rat testes of ages indicated. At day 24 meiotic divisions are seen and at day 40 late spermatids are present. In the 56-day-old rat mature spermatids are common.

Table 1. Body and testes weights of rats treated with DEHP in the diet for 14 days[a]

Age at start, days	Dosage, g/kg[b]	n	Body weight, g	Weight gain, g	Testes weight, g
25	Control	6	194 ± 6	114 ± 6	1.85 ± 0.05
	1.0	7	152 ± 8	86 ± 8	1.47 ± 0.16
	1.7	10	98 ± 7	27 ± 7	0.39 ± 0.06
40	Control	6	301 ± 7	105 ± 8	2.99 ± 0.09
	1.0	7	278 ± 14	90 ± 10	3.01 ± 0.37
	1.7	7	236 ± 14	52 ± 12	1.65 ± 0.75
60	Control	6	401 ± 14	51 ± 12	3.84 ± 0.11
	1.0	7	295 ± 4	−1 ± 8	3.43 ± 0.11
	1.7	6	340 ± 12	−1 ± 12	3.53 ± 0.16

[a] Values are means ± SD.
[b] Mean daily intake.

From this brief description of the development of the testis it can be concluded that it is of utmost importance to consider the age of the animals when using them in toxicity studies. If the exact age of the rat is not known proper evaluation of the testicular effects will be difficult. Moreover, when extrapolating data from rats to man it must be borne in mind that germ cell proliferation in the rat starts very early; the first meiotic cells appear within 2 weeks after birth. In humans, spermatogenesis starts at puberty (12–14 years of age). The term puberty, which is well defined in humans (*21*), should not be used in the rat, since there is no general agreement on the definition. In fact, if the situation in humans is translated to rats regarding testicular development, the rats are almost born in puberty. Consequently when data obtained in the rat are used for evaluation of the risk of testicular damage in childhood these fundamental differences should be considered.

Oral Administration of DEHP and Influence of Age

Gray and Butterworth (*8*) reported that oral administration of DEHP (2.8 g/kg/day for 10 days) to 4-, 10-, and 15-weeks-old Wistar rats produced testicular damage in the two younger groups but not in the older group. In an attempt to further elucidate this age-dependent difference in response, we treated groups of Sprague-Dawley rats (25, 40, and 60 days old at the beginning of the treatment) with DEHP. DEHP was administered in the diet and the dose was adjusted to give a daily intake of 1.0 or 1.7 g/kg body weight for 14 days. In this study we found that body weight gain was retarded in all groups and that testicular weight was markedly reduced in 25- and 40-day-old rats given 1.7 g/kg (Table 1). In these animals there was severe testicular damage, all tubules being affected in the 25-day-old rats (Fig. 2). When the mean daily intake was 1.0 g/kg, only a few tubules (1–10%) in each animal were affected. No clear changes were observed in the testes of mature rats at either dose of DEHP (Fig. 2). This mode of administration affected daily food intake, and therefore possible effects of the low food consumption were studied in pair-fed rats. Only minor testicular changes were observed in the pair-fed animals, excluding low food consumption as the only factor responsible for the testicular damage.

There may be several causes for the age-dependent

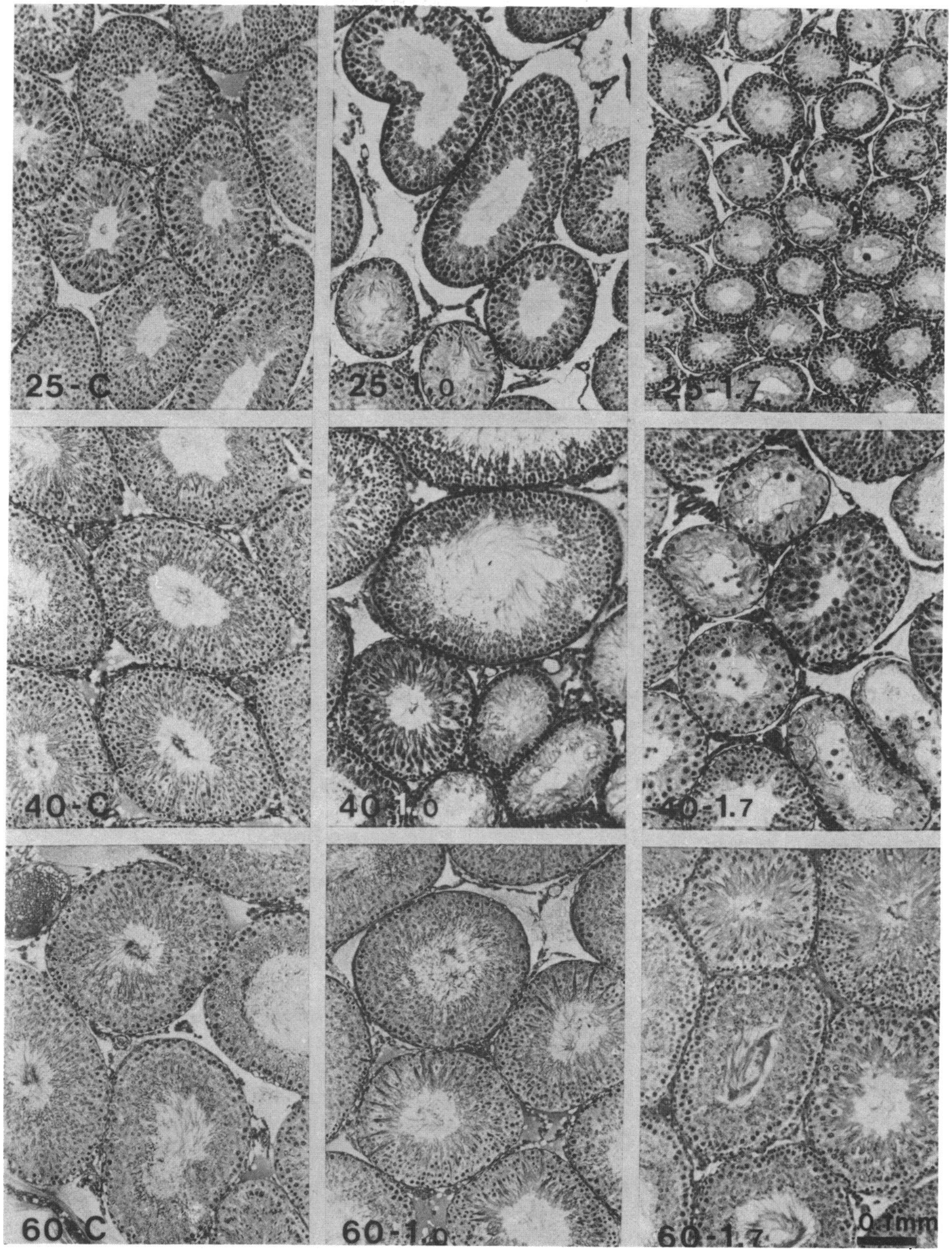

FIGURE 2. Series of light micrographs from the study where DEHP was given in the diet. Ages at start of dosing and doses are indicated. C = control. Note the severe damage in 25-day-old rats (1.7 g/kg/day) and partial damage in 25-day-old rats (1.0 g/kg/day) and 40-day-old rats (1.0 and 1.7 g/kg/day). There are no changes in 60-day-old animals. All micrographs at same magnification.

variation in testicular response to oral administration of DEHP. Gray and Beamand (*22*) investigated the possibility of a difference in tissue sensitivity using primary cultures of testicular cells. They found that germ cell detachment from the Sertoli cells was significantly higher in cultures from younger animals than in those from older animals when incubated with equal amounts of mono(2-ethylhexyl) phthalate (MEHP), the primary metabolite of DEHP. DEHP itself had no effect. It is at present difficult to relate these findings to the *in vivo* situation but they seem to suggest that there is an age-dependent difference in tissue sensitivity. However, spermatids, which in mature rats constitute most of the epithelium, are known to be lost during culture. Therefore, the testicular cell culture system may not adequately reflect the testicular epithelium of mature animals.

Another possible cause of the differing response between younger (immature) and older (mature) rats could

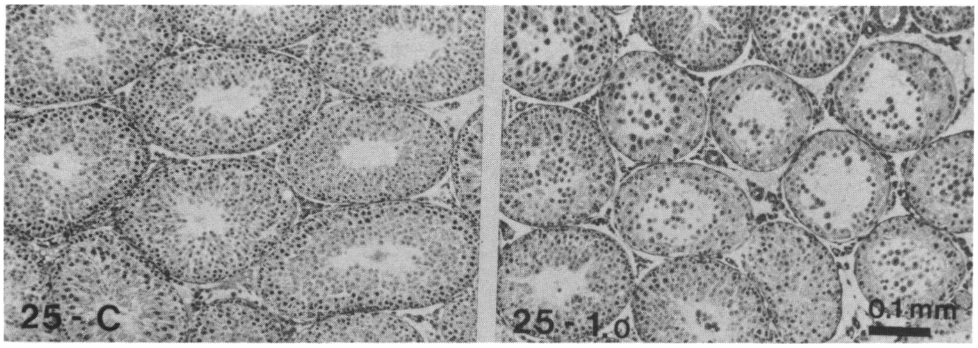

FIGURE 3. Light micrographs from testes of the 25-day-old groups to show the difference between DEHP-treated and control rats. Treatment was 1.0 g DEHP/kg/day by gavage for 14 days. Both micrographs at same magnification.

involve differences in absorption, distribution, metabolism, and/or excretion. To study this possibility and to overcome the problem of the reduced food intake, another series of experiments were carried out (*23*). Groups of rats (25, 40, and 60 days old) were given DEHP by gavage for 14 days at a dose of 1.0 g/kg/day. Animals that were 25 days old at the onset of the treatment showed severe testicular damage (Fig. 3), whereas the older animals were unaffected. In other animals, repeated blood samples were collected in order to estimate the exposure to DEHP and its primary metabolite MEHP. DEHP was not found to any significant extent in peripheral plasma whereas there were high levels of MEHP. The mean area under the plasma concentration–time curve of MEHP in the youngest age group was twice as high as in the two older groups. Finally, 25- and 60-day-old rats were given carbonyl-^{14}C-DEHP (1.0 g/kg) to measure the amount of DEHP-derived metabolites excreted in urine. Twice as much radioactivity was found in the 25-day-old group. The results indicated that the extent of absorption and hence total exposure to MEHP and its metabolites was higher in the younger rats. Thus it seems probable that the age-related difference in testicular response after oral administration may, at least in part, be explained by age differences in the pharmacokinetics of DEHP.

Intravenous Administration of DEHP

As most toxicity studies aim at obtaining a better knowledge of possible risks for humans, it is of importance to consider the route of administration. In humans, it is generally believed that the highest exposure to DEHP occurs in connection with hemodialysis or transfusions of blood and blood products where DEHP is introduced directly into the blood stream. Considerable exposure to DEHP has been reported to occur in newborn infants undergoing exchange transfusions (*24*). To reveal possible age-related effects on the testis of repeated intravenous infusions of DEHP, in a preliminary study we gave 25- and 40-day-old rats infusions of 500 mg/kg body weight, the highest tolerable dose of the DEHP emulsion. The infusions lasted 3 hr and were given every other day for 11 days (in all, six infusions/animal). No changes could be detected in paraffin embedded testes, but in Epon sections for light and electron microscopy some degenerating primary spermatocytes were observed in the 40-day-old group dosed with 500 mg/kg/day (Fig. 4). Some alterations were also observed in the Sertoli cells. No such changes were found in the 25-day-old group.

The results of this preliminary study indicate that intravenous administration of DEHP does not cause age-dependent testicular response similar to that observed after oral administration, suggesting that testicular sensitivity to intravenous DEHP of 25-day-old rats is not higher than that of 40-day-old animals. To confirm this suggestion, further studies are needed since it is not known if the disposition of DEHP after intravenous infusion varies with age.

Discussion

It is well established that oral administration of high doses of DEHP to rats causes testicular lesions, and that their effects are more pronounced in young animals where spermatogenesis is not fully established. The Sertoli cells play an important role in the establishment and maintenance of the specific microenvironment of the adluminal compartment of the seminiferous epithelium and this is a prerequisite for normal spermatogenesis. Alterations in this microenvironment will severely affect the spermatocytes and the spermatids that reside in the adluminal compartment (*12*). Thus, any agent interfering with normal Sertoli cell function may secondarily affect spermatogenesis. In this context it should be emphasized that an absence of morphological alterations in the Sertoli cells does not necessarily imply functional normality.

There are several reports indicating that the Sertoli cells are the cells primarily affected by treatment with phthalic acid esters (*25–27*), and so far there is no evidence of direct effects on the germ cell. Assuming that the Sertoli cells are "target cells" for the toxic metabolite(s) of DEHP, the seemingly contradictory results obtained after different modes of administration might in fact be explained by differences in the exposure of Sertoli cells and/or age differences in the metabolic activity of these cells. As previously pointed out, the result of our study with oral administration of DEHP suggested that the total exposure of MEHP and its metabolites was higher in 25-day-old rats than in more mature animals,

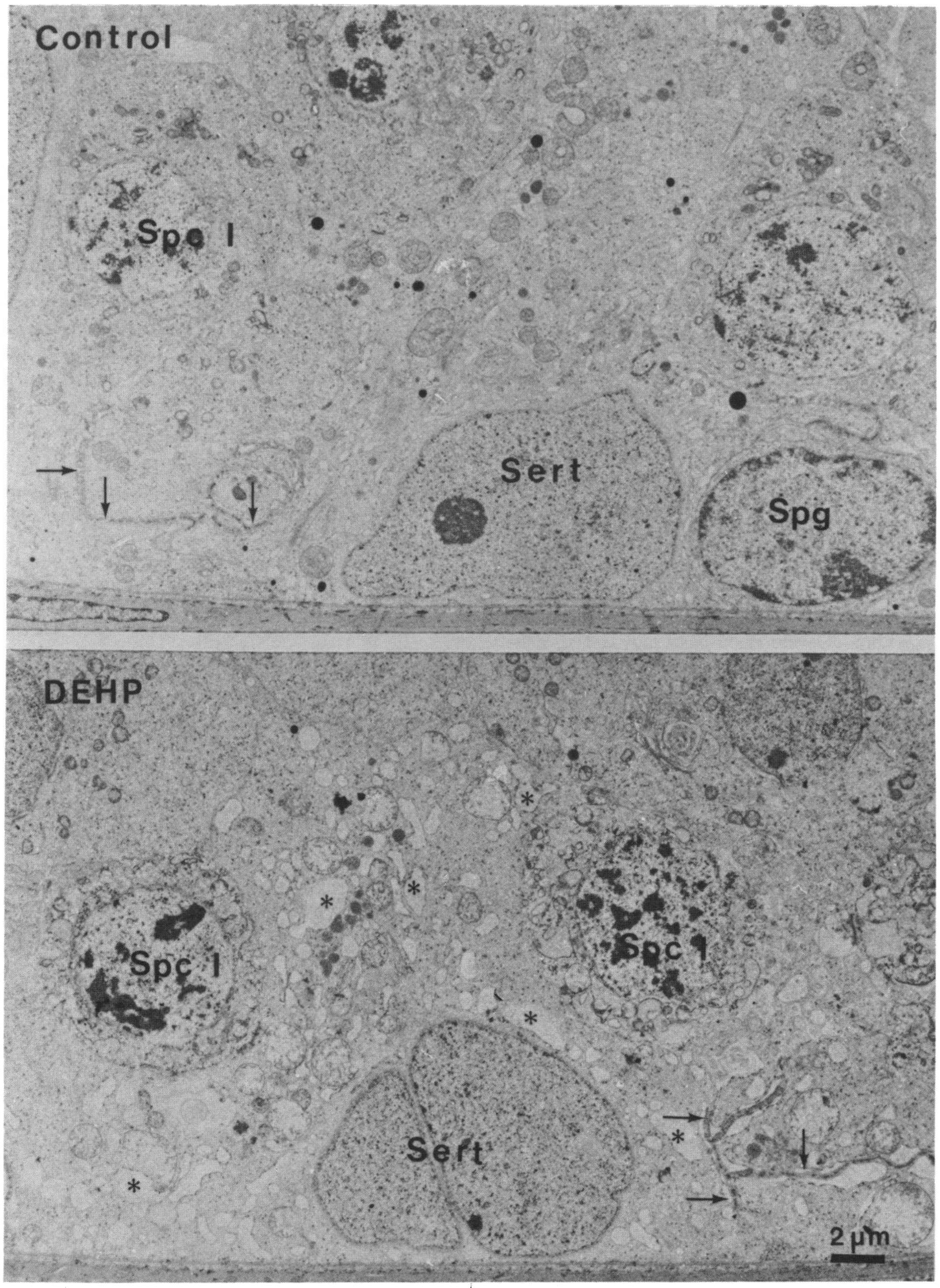

FIGURE 4. **Electron micrographs from 40-day-old rats in the infusion study. Note dilated endoplasmic reticulum (*) in the Sertoli cells** (Sert) of the animal given 500 mg DEHP/kg body weight. Note also the primary spermatocytes (Spc I) with signs of degeneration. Arrows point to seemingly normal inter-Sertoli cell junctions. Spg = spermatogonia.

which might account for the age difference in response. The minor differences found in the infusion study with some lesions in 40-day-old but not in 25-day-old rats, could be due to a somewhat higher "target cell" exposure in the older rats, since in these animals blood flow to the testis is higher but the number of Sertoli cells is the same as in the younger rats (*28*). Moreover, the Sertoli cells of older animals have a higher metabolic activity since they "support" more germ cells, and thus they may be more susceptible. A higher "target cell" exposure in the more mature animals could also result from age differences in the disposition of DEHP after intravenous administration.

Conclusions

Oral administration of high doses of DEHP to male rats of different ages produces more pronounced testicular damage in young rats. This difference in response seems to be due to a higher absorption of the DEHP-derived metabolite, MEHP, in the young animals. After intravenous infusions of DEHP, the younger rats were not more susceptible than the older ones. To assess the risk for testicular damage of DEHP exposure during childhood requires additional studies with species in which testicular development is more similar to that of humans.

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